JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

ESTROGEN RECEPTORS mRNA EXPRESSION IN THE PREHIERARCHICAL OVARIAN FOLLICLES IN THE GROWING CHICKEN AFTER GROWTH HORMONE ADMINISTRATION

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ARTICLE INFO

Received 25. 10. 2013
Revised 28. 11. 2013
Accepted 16. 12. 2013
Published 1. 2. 2014

Regular article

OPEN ACCESS

ABSTRACT

The previous study revealed that growth hormone (GH) affects steroidogenesis, proliferation and apoptosis in the ovary of the domestic hen. However, the mechanisms of GH action in the chicken ovary have not been fully elucidated. Thus, the current study was conducted to investigate the effect of GH on the mRNA expression of alfa (ERα) and beta (ERβ) estrogen receptors in the prehierarchical follicles of the chicken during puberty by RT-PCR method. The experiment was carried out on sexually immature 10-week-old Hy-Line hens that were injected with recombinant chicken GH (cGH) (experimental group) or vehicle (control group). The ovaries were isolated from birds in 2-weeks intervals from 10 to 16 weeks of age, and at 17 weeks, just after the onset of laying. Subsequently, the stroma, the white and the yellowish follicles were collected from the ovaries. GH significantly elevated the expression of ERα and ERβ mRNA in the white follicles of 16- and 17-week-old chickens as well as increased the expression of ERβ mRNA in the yellowish follicles after the onset of egg laying. In conclusion, the results of the current study demonstrated that the effect of GH on the expression of both forms of ERs was dependent on the size of follicle and the age of birds. It is suggested that GH via alternation of ERs gene expression in the prehierarchical follicles may participate in the chicken ovary development and functions.

Keywords: Growth hormone, estrogen receptors, ovary, chicken

INTRODUCTION

The domestic hen ovary contains several groups of follicles categorized with respect to their maturity. The most numerous is a group of growing prehierarchical follicles arranged according to their size into: primordial follicles (<1 mm in diameter) embedded in the ovarian stroma, white follicles (1-4 mm) and yellowish follicles (4-8 mm). Only very few of the small follicles are selected into a preovulatory hierarchy whereas the rest of them is the subject to the multihormonally controlled process of follicular atresia.

Ovarian stroma and prehierarchical follicles are a major source of estrogens within the chicken ovary **(Huang *et al*., 1979; Hrabia *et al*., 2004; Sechman *et al*., 2004)**. These hormones have a profound effect on the development and functions of the reproductive system in birds. Estrogens are known to affect the synthesis of egg yolk proteins in the liver and egg white proteins in the oviduct. Moreover, a growing body of evidence suggests that estrogens exhibit tissue protective effects in various organs including ovary.

Estrogens exert their impact on tissues via intracellular receptor-mediated mechanisms that modulate the transcription of specific genes. These hormones are known to act through two types of intracellular receptors, alfa (ERα) and beta (ERβ) **(Segars and Diggers, 2002)**. The estrogen receptors possess three major functional domains and act predominantly as ligand-activated transcription factors **(Hall *et al*., 2001; Segars and Diggers, 2002)**. Furthermore, ligand-dependent or ligand-independent ER activation have been reported to be accomplished by substances other than estrogens, for example cAMP, dopamine, and growth factors (**Segars and Diggers, 2002**). The expression of ERs mRNA has been evidenced in all the compartments of the chicken ovary **(Hrabia *et al*., 2008a)**.

In the recent years, it has also been demonstrated that growth hormone (GH) **(Hrabia *et al*., 2008b; Ahumada-Solorzano *et al*., 2012)** and its receptors **(Heck *et al*., 2003; Lebedeva *et al*., 2004; Hrabia *et al*., 2008b)** are present in all the compartments of the chicken ovary both during puberty and after the onset of egg laying indicating a local action of GH in this organ. The subsequent investigation of **Hrabia *et al.* (2011)** has revealed that GH administration elevated the weight of the ovary, the number of ovarian follicles as well as altered proliferation and apoptosis in the ovary of growing chickens. What is more, an increased content of estradiol and progesterone in the ovary of GH-treated chickens around the time of sexual maturity has been observed. Furthermore, the *in vitro* study of **Hrabia *et al.* (2012)** has demonstrated a stimulatory effect of GH on estradiol secretion by isolated prehierarchical (white and yellowish) follicles, whereas **Ahumada-Solorzano *et al.* (2012)** have reported that GH increased progesterone synthesis by the granulosa cells of yellow preovulatory follicles. However, the question if GH may exert its effects by altering the expression of ERs remains without answer. Therefore, the present study has been conducted to investigate whether exogenous GH affects ERα and ERβ mRNA expression in the stroma and prehierarchical follicles of growing chickens.

MATERIAL AND METHODS

All procedures were performed in accordance with the research protocols approved by the Local Animal Ethics Committee in Krakow, Poland.

Hy-Line chickens were purchased from the commercial farm Drobeco (Palowice, Poland) and caged individually under a photoperiod of 14L:10D with free access to water and commercial feed. At the age of 10 weeks birds were divided into control (n=30) and experimental group (n=30). The control group received subcutaneous injections of vehicle (0.05% BSA in 0.9% NaCl) three times a week, whereas experimental group was injected with 200 µg of recombinant chicken GH (cGH) per kg of body weight. The birds were killed by decapitation in 2-weeks intervals from 10 to 16 weeks of age and at 17 weeks, just after the onset of egg laying. Subsequently, following ovarian compartments were isolated: stroma with primordial follicles <1 mm in diameter (STR), white follicles (>1-4 mm; WF) and yellowish follicles (>4-8 mm; YF). After removal of existing yolk from the follicles, the samples were immediately placed in RNAlater and stored at -20°C until total RNA extraction.

Total RNA isolation and RT-PCR analysis

Total RNA was extracted from the ovarian tissues using TRI-reagent according to manufacturer's recommendation (MRC, Inc.,USA). Total RNAs (5 µg) from each tissue were reverse-transcribed with RevertAid M-MuLV reverse transcriptase (200 U) (Fermentas, Lithuania) and oligo-dT_{18} primers (0.5 µg). As a negative control untranscribed tissue RNA (reverse transcriptase omitted) was used. RT products (1 µl) were amplified in a Thermocycler Gradient (Eppendorf, Germany) in a 12.5 µl of reaction mixture containing 1.25 µl of buffer (100 mmol Tris-HCl, pH 8.8, 500 mmol KCl, 0.8% Nonidet P40), 0.312 unit pol Taq DNA polymerase, 0.2 µmol sense and antisense primers, 0.2 mmol each dNTP, 1.5

mmol $MgCl_2$, and water (Fermentas, Lithuania). After the initial denaturation for 5 min at 95^0C (ERα, 18S) or 4 min at 94^0C (ERβ), the amplification profiles were applied as shown in Table 1. Amplifications were completed with an additional extension at 72^0C for 7 min. Negative control (water) was included in all reactions. The primers for ERα, ERβ and 18S rRNA are describe in Table 1. All PCR products were electrophoresed in 1.5% agarose gel containing ethidium bromide in 0.5x TBE buffer. The gel was examined under UV light and photographed with a digital camera. The net intensities of individual bands were measured using the Scion Image for Windows. The ratios of net intensity of examined genes to 18S rRNA were used to represent the relative level of target gene expression. The average abundance of six repeats was used for statistical analysis.

Relative expressions of ERα and ERβ were expressed as mean ± SEM, and the significance of their differences between the control and cGH treated groups were examined by Student *t*-test. Differences were considered significant at $P<0.05$.

Table 1 Characteristics of primers and PCR conditions used in this study

Gene	GenBank	Primer sequence	PCR product	PCR conditions
ERα	X03805	F: 5'-GTGCCTTAAGTCCATCATCCT-3' R: 5'-GCGTCCAGCATCTCCAGTAAG-3'	300 bp(1522-1821)	95°C 30s, 58°C 30s, 72°C 30s, 30 cycles
ERβ	AB036415	F: 5'-TGATATGCTCCTGGCCATGAC-3' R: 5'-CTTCATGCTCAGCAGATGCTC-3'	304 bp(1384-1657)	94°C 30s, 55°C 30s, 72°C 30s, 30 cycles
18S rRNA	AF173612	F: 5'-CGCGTGCATTTATCAGACCA-3' R: 5'-ACCCGTGGTCACCATGGTA-3'	167 bp(160-326)	94°C 30s, 60°C 30s, 72°C 30s, 30 cycles

RESULTS AND DISCUSSION

As the chicken ovary is a target tissue for GH action, as well as an organ where sex steroids are synthesized and act, it represents a potential site of regulatory interaction between GH and steroids. Thus, to find cooperation between GH and estrogen action in the chicken ovary the expression of ERα and ERβ mRNA after GH administration during maturation was investigated. The examined ERs were found in all analyzed ovarian compartments during puberty. The products were 300, 304 and 167 bp for ERα mRNA, ERβ mRNA and 18S rRNA mRNA, respectively, and corresponded to the approximate size for each as predicted.

It was found that the relative expression of ERα mRNA was higher than that of ERβ mRNA. It is consistent with the previous results showing distribution of both forms of ERs in all the compartments of the chicken ovary **(Hrabia *et al*., 2008a)**. In contrary, mammalian ovaries are organs with higher expression of ERβ **(Słomczyńska and Woźniak 2001; Nynca *et al.* 2013)**. Administration of cGH did not significantly change the ERα mRNA expression in the stroma with primordial follicles at any examined period of sexual maturation (Fig. 1A). In the white follicles that appeared in the ovary at the age of 14 weeks, GH significantly elevated the relative expression of ERα mRNA in 16- and 17-week-old chickens by 109% and 88%, respectively (Fig. 1B). In the yellowish follicles present in the ovary from 16 weeks of age GH did not affect ERα mRNA expression (Fig. 1C). Previously, it was observed that GH stimulates proliferation and inhibits apoptosis in the prehierarchical follicles of the growing chicken ovary **(Hrabia *et al*., 2011)**, and ERα is thought to be involved in regulation of the processes of follicular atresia and proliferation. Thus, GH by increasing ERα mRNA expression might regulate apoptosis in the avian ovarian follicles.

The present study did not reveal any significant differences in the relative expression of ERβ mRNA in the stroma between the control and GH-treated groups during chicken sexual maturation (Fig. 2A), whereas GH administration significantly increased the expression of ERβ mRNA in the white follicles in 16- and 17-week-old chickens, by 174% and 77%, respectively (Fig. 2B). The ERβ mRNA expression was also markedly elevated in the yellowish follicles by 129% in 16-week-old GH-treated chickens when compared to the control hens (Fig. 2C). Within the hen ovary, prehierarchical follicles are known to possess a great steroidogenic potential and ERβ is considered to be a mediator of estrogens in regulation of local steroidogenesis **(Britt and Findlay, 2002)**. On the other hand, exogenous GH increases the estradiol and progesterone content in the chicken ovary during puberty **(Hrabia *et al*., 2011)** and *in vitro* stimulates estradiol secretion by prehierarchical follicles **(Hrabia *et al*., 2012)**. Therefore, it seems possible that the follicular response to GH is associated with ERβ gene expression and in consequence steroids production.

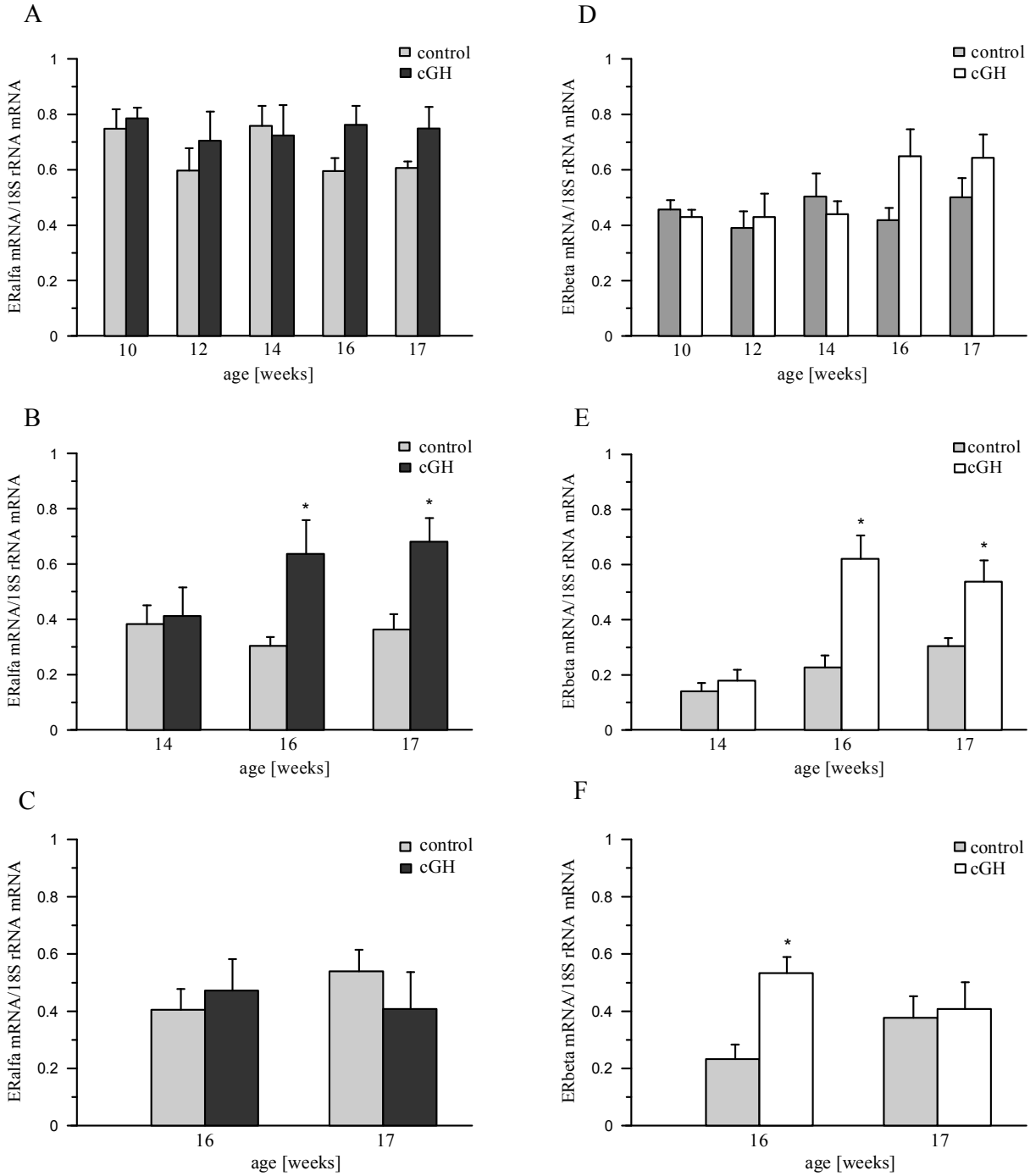

Figure 1 Effect of chicken growth hormone on ERα (A, B, C) and ERβ (D, E, F) mRNA expression in the chicken ovarian stroma, white follicles and yellowish follicles during sexual maturation determined by RT-PCR. STR – stroma, WF – white follicles, YF – yellowish follicles, cGH – experimental group. Each value represents the mean ± SEM from 6 determinations; *P<0.05 – compared to control group.

CONCLUSION

The results of the current study demonstrated that the effect of GH on the expression of ERα and ERβ mRNA was dependent on the size of follicle and the age of birds. It is suggested that GH via alteration of ERs gene expression in the prehierarchical follicles may participate in the chicken ovary development and functions.

Acknowledgments: The authors wish to thank Prof. Arieh Gertler (Institute of Biochemistry, Food Science and Nutrition, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot, Israel) for the generous gift of chicken GH. The study was supported by the quality grant for PhD students and DS-3243/KFiEZ.

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